Bottom-up versus top-down induction of sleep by zolpidem acting on histaminergic and neocortex neurons

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Abstract: Zolpidem, a GABAA receptor-positive modulator, is the gold-standard drug for treating insomnia. Zolpidem prolongs IPSCs to decrease sleep latency and increase sleep time, effects that depend on 2 and/or 3 subunit-containing receptors. Compared with natural NREM sleep, zolpidem also decreases the EEG power, an effect that depends on 1 subunit-containing receptors, and which may make zolpidem-induced sleep less optimal. In this paper, we investigate whether zolpidem needs to potentiate only particular GABAergic pathways to induce sleep without reducing EEG power. Mice with a knock-in F77I mutation in the GABAA receptor 2 subunit gene are zolpidem-insensitive. Using these mice, GABAA receptors in the frontal motor neocortex and hypothalamic (tuberomammillary nucleus) histaminergic-neurons of 2I77 mice were made selectively sensitive to zolpidem by genetically swapping the 2I77 subunits with 2F77 subunits. When histamine neurons were made selectively zolpidem-sensitive, systemic administration of zolpidem shortened sleep latency and increased sleep time. But in contrast to the effect of zolpidem on wild-type mice, the power in the EEG spectra of NREM sleep was not decreased, suggesting that these EEG power-reducing effects of zolpidem do not depend on reduced histamine release. Selective potentiation of GABAA receptors in the frontal cortex by systemic zolpidem administration also reduced sleep latency, but less so than for histamine neurons. These results could help with the design of new sedatives that induce a more natural sleep.

DOI: https://doi.org/10.1523/JNEUROSCI.3714-15.2016

Systems/Circuits

Bottom-Up versus Top-Down Induction of Sleep by Zolpidem Acting on Histaminergic and Neocortex Neurons

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Zolpidem, a $GABA_A$ receptor-positive modulator, is the gold-standard drug for treating insomnia. Zolpidem prolongs IPSCs to decrease sleep latency and increase sleep time, effects that depend on α2 and/or α3 subunit-containing receptors. Compared with natural NREM sleep, zolpidem also decreases the EEG power, an effect that depends on α1 subunit-containing receptors, and which may make zolpidem-induced sleep less optimal. In this paper, we investigate whether zolpidem needs to potentiate only particular GABAergic pathways to induce sleep without reducing EEG power. Mice with a knock-in F77I mutation in the $GABA_A$ receptor γ2 subunit gene are zolpidem-insensitive. Using these mice, $GABA_A$ receptors in the frontal motor neocortex and hypothalamic (tuberomammillary nucleus) histaminergic-neurons of γ2I77 mice were made selectively sensitive to zolpidem by genetically swapping the γ2I77 subunits with γ2F77 subunits. When histamine neurons were made selectively zolpidem-sensitive, systemic administration of zolpidem shortened sleep latency and increased sleep time. But in contrast to the effect of zolpidem on wild-type mice, the power in the EEG spectra of NREM sleep was not decreased, suggesting that these EEG power-reducing effects of zolpidem do not depend on reduced histamine release. Selective potentiation of $GABA_A$ receptors in the frontal cortex by systemic zolpidem administration also reduced sleep latency, but less so than for histamine neurons. These results could help with the design of new sedatives that induce a more natural sleep.

Key words: GABA-A receptor; histamine; insomnia; sleep; tuberomammillary nucleus; zolpidem

Significance Statement

Many people who find it hard to get to sleep take sedatives. Zolpidem (Ambien) is the most widely prescribed "sleeping pill." It makes the inhibitory neurotransmitter GABA work better at its receptors throughout the brain. The sleep induced by zolpidem does not resemble natural sleep because it produces a lower power in the brain waves that occur while we are sleeping. We show using mouse genetics that zolpidem only needs to work on specific parts and cell types of the brain, including histamine neurons in the hypothalamus, to induce sleep but without reducing the power of the sleep. This knowledge could help in the design of sleeping pills that induce a more natural sleep.

Introduction

Many healthy people who cannot sleep, as well as many with neurological or mental illness, take sedatives (Wafford and Ebert, 2008; Winsky-Sommerer, 2009; Nutt and Stahl, 2010; Mignot, 2013; Rihel and Schier, 2013). Zolpidem (Ambien), a nonbenzodiazepine $GABA_A$ receptor-positive modulator, is currently the most successful "sleeping pill" (Nutt and Stahl, 2010). In the United States alone, there were 53.4 million prescriptions for

Received Oct. 9, 2015; revised Aug. 16, 2016; accepted Aug. 17, 2016.

Author contributions: D.S.U., Z.Y., S.G.B., N.P.F., and W.W. designed research; D.S.U., Z.Y., A.Y.Z., E.C.H., X.Y., and R.Y. performed research; X.Y. and A.L.V. contributed unpublished reagents/analytic tools; D.S.U., Z.Y., A.Y.Z., S.G.B., N.P.F., and W.W. analyzed data; D.S.U., Z.Y., S.G.B., N.P.F., and W.W. wrote the paper.

This work was supported by Biotechnology and Biological Sciences Research Council studentship to D.S.U., Biotechnology and Biological Sciences Research Council Grant BB/K018159/1 to S.G.B., N.P.F., and W.W., Medical Research Council Grant G0901892 to S.G.B., N.P.F., and W.W., Medical Research Council Grant G0800399 to W.W., Wellcome Trust WT094211MA to S.G.B., N.P.F., and W.W., Joint Investigator Award 107839/Z/15/Z to N.P.F., Joint Investigator Award 107841/Z/15/Z to W.W., Biotechnology and Biological Sciences Research Council Doctoral Training Grant BB/F017324/1 to E.C.H., and a United Kingdom-China Scholarships for Excellence/China Scholarship scheme to X.Y. and Z.Y.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.3714-15.2016

Table 1. Time and percentage of time spent in Wake, NREM, and REM sleep for the different types of mice used in the study[a]

	Wake			NREM			REM		
Genotype	Time (min)	%	SEM	Time (min)	%	SEM	Time (min)	%	SEM
C57BL/6J	68.83	57.88	10.38	44.25	37.17	8.00	5.92	4.96	3.01
γ2I77^{lox}	64.00	54.92	4.68	46.50	39.72	4.47	6.33	5.36	1.74
HDC-γ2F77	71.58	60.72	5.86	39.08	33.23	5.96	7.08	6.05	1.56
FC-γ2F77	54.83	47.08	3.48	54.50	46.68	3.50	7.33	6.24	1.38
SC-γ2F77	64.17	56.12	6.21	46.21	40.45	0.97	3.92	3.43	1.73

[a]The data were recorded over a baseline period of 2 h. One-way ANOVA revealed no significant differences between the mouse types for Wake, NREM, or REM sleep.

zolpidem in 2010 (Greenblatt and Roth, 2012). The drug decreases sleep latency, the time to the onset of NREM sleep (Arbilla et al., 1985; Gottesmann et al., 1998; Alexandre et al., 2008; Anaclet et al., 2012; Xu et al., 2014). After taking a 10 mg tablet of zolpidem, the average person goes to sleep after ~12 min (Greenblatt and Roth, 2012). Compared with natural (drug-free) NREM sleep, however, the NREM sleep induced by zolpidem has reduced power in most EEG frequencies (Landolt et al., 2000; Kopp et al., 2004; Alexandre et al., 2008). This reduced power may indicate that zolpidem-induced sleep is suboptimal compared with natural NREM sleep.

Zolpidem works at three $GABA_A$ receptor subtypes: $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$, and $\alpha3\beta\gamma2$ (Pritchett and Seeburg, 1990; Crestani et al., 2000; Cope et al., 2004; Kopp et al., 2004; Leppä et al., 2011), and has the highest affinity at $\alpha1$-containing receptors (Pritchett and Seeburg, 1990). These $\alpha1\beta\gamma2$-containing receptors are expressed widely (Niddam et al., 1987; Wisden et al., 1988, 1992; Pritchett et al., 1989; Duncan et al., 1995; Fritschy and Mohler, 1995; Hörtnagl et al., 2013). Surprisingly, the $\alpha1$-containing GABA receptors are not responsible for zolpidem's ability to promote sleep. In mice with $\alpha1$ subunits made insensitive to zolpidem by a mutation, H101R, zolpidem reduces latency to NREM sleep and prolongs NREM sleep time as well as it does in wild-type mice (Kopp et al., 2004). Thus, zolpidem's sleep-promoting effects come from enhancing GABA's actions at $GABA_A$ receptors with $\alpha2$ and/or $\alpha3$ subunits. Instead, the $\alpha1$H101R mice do show that $\alpha1$-containing receptors are needed for zolpidem to produce its characteristic decrease in the EEG power in NREM sleep (Kopp et al., 2004). But can zolpidem potentiate $\alpha2$ and/or $\alpha3$-containing receptors in only particular GABA pathways to induce sleep without reducing EEG power? The answer could help design sedatives that produce a more natural sleep.

Natural NREM sleep is hypothesized to start when GABA neurons in the preoptic hypothalamus increase their activity onto, among other targets, the wake-promoting histaminergic neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamus (Nitz and Siegel, 1996; Sherin et al., 1996, 1998; Zhang et al., 2015). Infusing the $GABA_A$ agonist muscimol into this area induces sleep (Lin et al., 1989; Nitz and Siegel, 1996); conversely, injecting $GABA_A$ receptor antagonists there decreases the potency of GABAergic anesthetics (Nelson et al., 2002). Thus, $GABA_A$ receptor modulators, such as zolpidem, could produce sleep by decreasing histaminergic activity (Nelson et al., 2002). Histaminergic neurons express $\alpha1\beta3\gamma2$ and $\alpha2\beta3\gamma2$ $GABA_A$ receptors (Fritschy and Mohler, 1995; Sergeeva et al., 2002; Zecharia et al., 2009, 2012; May et al., 2013); and so, $GABA_A$ receptors on these neurons could, in part, account either for the EEG power-reducing effect of zolpidem ($\alpha1$-containing receptors), or critically, the ability of zolpidem to reduce latency to NREM sleep and increase sleep time ($\alpha2$-containing receptors).

We previously used a pharmacogenetics method, based on mutated $GABA_A$ receptors, for probing how endogenous GABA inputs onto selected cell types generates behavior (Wulff et al., 2007; Wisden et al., 2009; Sieghart, 2012). The mutation F77I in the $\gamma2$ subunit ($\gamma2$I77) abolishes zolpidem binding to $GABA_A$ receptors (Buhr et al., 1997; Wingrove et al., 1997; Cope et al., 2004). In $\gamma2I77^{lox}$ mice, the zolpidem-insensitive $\gamma2$I77 subunits can be swapped with zolpidem-sensitive $\gamma2$F77 versions (Wulff et al., 2007). Here, using this method, we found that strengthening inhibition onto histamine neurons by zolpidem induces NREM sleep but does not reduce EEG power.

Materials and Methods

Mice. All experiments were performed in accordance with the United Kingdom Home Office Animal Procedures Act (1986); all procedures were approved by the Imperial College Ethical Review Committee. All mice weighed between 19 and 30 g and were ~17 weeks old at the time of AAV injections. Both male and female mice were used, but no sex differences were observed and the data were pooled. The sleep–wake studies and drug administrations were started ~4 weeks after AAV injection (see below). Adult *C57BL/6J* mice were purchased from Harlan. The *HDC-Δγ2I77* mice were produced by crossing *HDC-Cre (JAX* stock #021198, RRID:IMSR_JAX:021198) and *γ2I77^{lox}*- (zolpidem-insensitive) mice (JAX stock #021197, RRID:IMSR_JAX:021197) on *C57BL/6J* backgrounds, as described previously (Zecharia et al., 2012). In adult *HDC-Cre* mice, Cre recombinase expression is driven by the endogenous *hdc* gene and is found selectively in histaminergic neurons in the TMN, and mast cells in the rest of the brain; the knock-in *HDC-Cre* allele expresses functional HDC protein (Zecharia et al., 2012). In the *γ2I77^{lox}*-mice, exon 4 of the $GABA_A$ receptor $\gamma2$ subunit gene (*gabrg2*), which encodes the critical I77 residue, is flanked by *loxP* sites (Wulff et al., 2007); deletion of exon 4 by Cre recombinase produces a null *gabrg2* allele (Wulff et al., 2007, 2009a, b; Rovó et al., 2014). The baseline vigilance-state data (% Wake, NREM, and REM) recorded for a 2 h period, as determined by EEG/EMG scoring, for the mice in drug-free conditions are shown in Table 1 (see EEG recordings and sleep scoring).

Generation of HDC-γ2F77, FC-γ2F77, *and superior colliculi (SC)* SC-γ2F77 *mice.* Stereotaxic injections of AAV were performed with a Leica Angle Two frame under isoflurane anesthesia, using Hamilton microliter #701 10 μl syringes with adjoining capillary glass pipettes tapered 1 mm to ~50 μm diameter, back-loaded with mineral oil and AAV mixture (1:1 with 20% mannitol) in the tip (see AAV transgenes and AAV production). The coordinates of the (bilateral) injection sites according to the digital atlas of the Leica apparatus were, relative to bregma: frontal cortex (FC) of $\gamma2I77^{lox}$ mice (mediolateral ±1.75, anteroposterior 1.87, dorsoventral −1.70 (mm), 1.5 μl *AAV-Cre-2A-γ2F77* and *AAV-flex-rev-EGFP* suspension per side, with the two AAVs mixed 1:1 before injection); SC of *γ2I77^{lox}* mice (mediolateral ±1, anteroposterior −3.88, dorsoventral −2 (mm), 1 μl *AAV-Cre-2A-γ2F77* suspension per side); TMN of *HDC-Δγ2I77* mice (mediolateral ±0.92, anteroposterior −2.70, dorsoventral −5.34 (mm), 1.5 μl AAV- *flex-rev-γ2F77-2A-Venus* suspension per side). After AAV injections, the mice recovered for a minimum of 4 weeks in their home cages. The baseline vigilance-state data (% Wake, NREM, and REM), as determined by EEG/EMG scoring, for the *HDC-γ2F77*, *FC-γ2F77*, and *SC-γ2F77* mice in drug-free conditions for a 2 h period are shown in Table 1 (see EEG recordings and sleep scoring).

AAV transgenes and AAV production. The *AAV-panpromoter-flex-rev-Venus-2A-γ2F77* transgene was constructed from components of *pAAV-CAG-promoter-Cre-2A-γ2F77,* kindly provided by Zoltan Nusser,

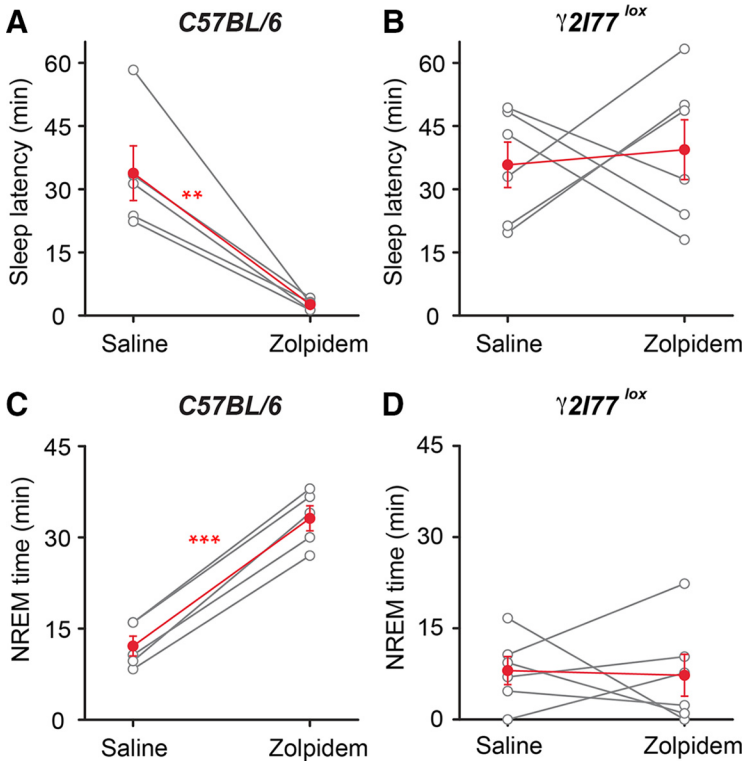


Figure 1. Zolpidem reduces sleep latency and increases sleep time in *C57BL/6J* but not in $\gamma 2I77^{lox}$ mice. Sleep latencies after saline and zolpidem (5 mg/kg) injections in *C57BL/6J* (***A***) and $\gamma 2I77^{lox}$ (***B***) mice. For *C57BL/6J* mice: $^{**}p = 0.008$ (paired *t* test). ***C***, Time spent in NREM sleep in the first 45 min after saline and zolpidem in *C57BL/6J* (***C***) and $\gamma 2I77^{lox}$ (***D***) mice. For *C57BL/6J* mice: $^{***}p = 0.0003$ (paired *t* test).

Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary (Sumegi et al., 2012); and *pAAV-flex-rev-hM4-mCherry* (Addgene plasmid 44362, gift of Bryan Roth, University of North Carolina at Chapel Hill, NC (Krashes et al., 2011), Addgene, RRID:SCR_002037). The "panpromoter" in the *AAV-panpromoter-flex-rev-γ2F77-2A-Venus* transgene was derived from a fragment of the promoter of the mouse histidine decarboxylase (*hdc*) gene; we originally hoped that this *hdc* promoter fragment would be selective for histaminergic cells but found that it worked well in all neuronal cell types we tested (unpublished data). The *AAV-panpromoter-flex-rev-γ2F77-2A-Venus* transgene plasmid has been deposited at Addgene (Addgene Plasmid 71410, Addgene, RRID:SCR_002037). The *AAV-flex-rev-EGFP* transgene was Addgene plasmid 28304 (gift from Edward Boyden, Massachusetts Institute of Technology, Cambridge, MA). The AAV transgenes *AAV-flex-rev-γ2F77-2A-Venus*, *AAV-Cre-2A-γ2F77*, and *AAV-flex-rev-EGFP* were each packaged in AAV1/2 capsids and purified with heparin columns (Klugmann et al., 2005). AAV was suspended in sterile PBS at 1:1 concentration.

EEG recordings and sleep scoring. For nontethered EMG and EEG recordings, mice were fitted with Neurologger 2A devices (Anisimov et al., 2014). The recordings took place in home cages. Data were sampled at 200 Hz and downloaded offline and processed with Spike2 software (Cambridge Electronic Design, Spike2 Software, RRID:SCR_000903). The EEG was high-pass filtered (0.5 Hz corner frequency, −3 dB). The EMG was bandpass filtered (5–45 Hz, −3 db). Wake, non-REM, and REM were first automatically sleep-scored using previously described criteria (Costa-Miserachs et al., 2003). The EEG results were then manually verified.

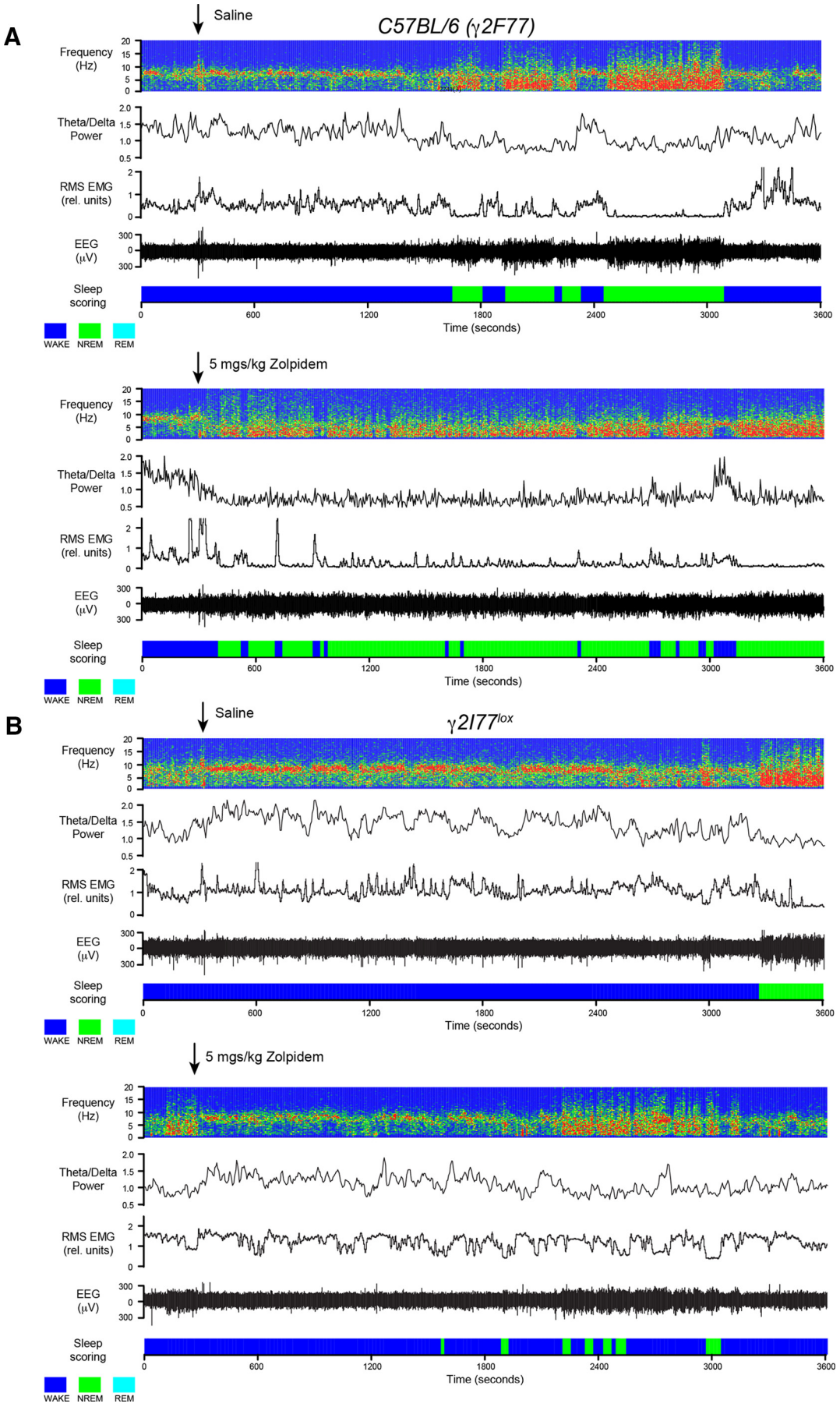
To calculate power spectra, segments of NREM identified after sleep scoring were concatenated and power spectra calculated using a Fast Fourier transform with a Hanning window function. Segments of data of at least 10 min were used. The power spectra were normalized as we described previously, such that the area under the saline controls for a given genotype was unity (Zhang et al., 2015). Power changes were computed as differences in areas under the power spectra.

Behavioral experiments and drug administration. Mice were maintained on a 12 h light/12 h dark cycle with *ad libitum* food and water. All behavioral experiments took place during the "lights off" part of the cycle when the mice were most active. In all cases, we used paired comparisons where the animals served as their own controls, a within-animals approach (crossover design). The experimenters were not blinded to treatment. Zolpidem (Tocris Biosciences) was dissolved in equimolar tartaric acid (BDH Chemicals) in 0.9% w/v saline.

Immunohistochemistry. Mice were anesthetized with pentobarbital and transcardially perfused with PBS followed by 4% PFA. Brains were submerged in 4% PFA overnight and stored in 30% sucrose. Free-floating sections (40 μm) were blocked with 10% normal goat serum, 1% BSA, 0.1% Triton X in PBS (2 h, room temperature). Sections were incubated for 24–48 h at 4°C with primary antisera: rabbit anti-Cre (1:1000; Novagen), or rabbit anti-GFP (1:1000; Stratagene), or rabbit streptavidin (1:1000; Alexa-Fluor-555 conjugate; Invitrogen). Secondary goat anti-rabbit antisera (Alexa-Fluor-488 or 594, 1:1000; Invitrogen) were incubated for 2 h at room temperature. Pictures were taken using a Nikon eclipse 80*i* microscope with Qcapture Pro software (Q Capture software, RRID:SCR_014432), or a Zeiss Cell Observer Live Cell Imaging System, or a Zeiss LSM 510 inverted microscope with Zen pro software. Images were processed using ImageJ (open source, ImageJ, RRID:SCR_003070) and Adobe Photoshop (Adobe Photoshop CS6, RRID:SCR_014199).

Quantifying the spread of AAV transduction. For the *HDC-γ2F77* mice, Venus-positive neurons were counted on fixed sections using ImageJ. Signal-emitting outliers (<15 μm or >30 μm diameter) were excluded, as were objects visually scored as incorrect (e.g., microglia). For the *FC-γ2F77* and *SC-γ2F77* mice, Cre-positive neurons were identified by immunohistochemistry, and the percentage area of the target region (FC or SC) was calculated, again using ImageJ (ImageJ, RRID:SCR_003070).

Brain-slice electrophysiology. We recorded spontaneous IPSCs from whole-cell, voltage-clamped, HDC neurons of the TMN and pyramidal neurons of the FC in acute slices. Brains were rapidly removed and immersed in ice-cold slicing ACSF (85 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 75 mM sucrose, 11 mM glucose, bubbled with 95% O_2/5% CO_2). For the TMN, a tissue block was cut between the cerebellum and optic tract, and coronal sections were cut to a thickness of 250 μm on a vibratome. For the FC, a tissue block was cut between the optic tract and ~1 mm behind the olfactory bulb. After slicing, the holding chamber was transferred to a 37°C heat block for 15 min, and slicing ACSF was gradually exchanged for recording ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 11 mM glucose, pH 7.4, bubbled with 95% O_2/5% CO_2) over 40 min. Electrophysiological recordings were made at room temperature. We identified histaminergic neurons by the presence of hyperpolarization-activated currents (*I*h), a transient outward current, and the spontaneous firing activity of the cells (Stevens et al., 2001). Virally transduced pyramidal neurons in the FC were found by Cre-dependent expression of EGFP. Pyramidal neurons were identified primarily by morphology. For detection of IPSCs, we performed whole-cell recordings in voltage-clamp (−70 mV) using internal pipette solutions containing the following: 140 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 10 mM


A
Saline
C57BL/6 (γ2F77)
Frequency (Hz)
Theta/Delta Power
RMS EMG (rel. units)
EEG (μV)
Sleep scoring
WAKE NREM REM
Time (seconds)
5 mgs/kg Zolpidem
B
Saline
γ2I77lox
5 mgs/kg Zolpidem

HEPES, 5 mM EGTA, and 2 mM Mg-ATP; the pH was adjusted to 7.3 with CsOH.

Statistical analysis. For behavioral and EEG comparisons, and comparisons of IPSC decay times from the electrophysiology analysis, we used two-tailed paired *t* tests (Janusonis, 2009), and normality was assumed. Analyses were performed in OriginPro 8.6 or GraphPad Prism 4.03 (GraphPad Prism, RRID:SCR_002798).

Results

Zolpidem does not induce sleep in γ2I77^{lox} mice

The wild-type γ2 subunit (γ2F77) confers zolpidem sensitivity on α1β, α2β, and α3β subunit-containing $GABA_A$ receptors (Pritchett et al., 1989; Pritchett and Seeburg, 1990; Günther et al., 1995). The binding site for zolpidem, at the interface between the α and γ2 subunits, requires residue F77 in the γ2 subunit (Buhr et al., 1997; Wingrove et al., 1997). Changing the F77 residue to I (F77I) abolishes zolpidem binding. Consequently, knock-in mice with the γ2 point mutation γ2F77I (*γ2I77^{lox}* mice) are behaviorally insensitive to zolpidem in the dose range 1–30 mg/kg (Cope et al., 2004; Wulff et al., 2007; Leppä et al., 2011). However, EEG and sleep responses of *γ2I77^{lox}* mice following zolpidem administration have not been investigated. *C57BL/6J* mice and *γ2I77^{lox}* mice showed no difference in their baseline sleep parameters (Table 1). We then established how zolpidem (5 mg/kg), compared with a saline injection, influenced latency to NREM-like sleep and total sleep time in *C57BL/6J* mice and *γ2I77^{lox}* mice. Zolpidem at 5 mg/kg (systemic injection, i.p.) caused *C57BL/6J* (γ2F77) mice to enter NREM sleep in 2.6 ± 0.6 min ($n = 5$; paired *t* test, $t_{(4)} = 4.9$, $p = 0.008$) (Fig. 1*A*), whereas at this dose of zolpidem, *γ2I77^{lox}* mice did not fall asleep for 39 ± 7 min ($n = 6$; paired *t* test, $t_{(5)} = 0.31$, $p = 0.77$) (Fig. 1*B*), which was approximately the same time they and *C57BL/6J* mice took to fall into NREM sleep following a saline injection (Fig. 1*A*). In *C57BL/6J* mice, in the first 45 min after injection, zolpidem more than doubled the amount of NREM-like sleep over baseline (from 12 ± 1.6 min to 33 ± 2 min; $n = 5$; paired *t* test $t_{(4)} = 20.77$, $p = 0.0003$; Fig. 1*C*). The effect persisted until at least 90 min after injection. These data, reduced sleep latency and prolonged sleep time, are consistent with previous reports on zolpidem's action in *C57BL/6J* mice (Kopp et al., 2004; Alexandre et al., 2008). By contrast, there was no change in the sleep time above baseline in zolpidem-injected *γ2I77^{lox}* mice ($n = 6$ paired *t* test, $t_{(5)} = 0.18$, $p = 0.86$; Fig. 1*D*). Examples of the primary EEG/EMG recordings for saline- and zolpidem-injected *C57BL/6 (γ2F77)* and *γ2I77^{lox}* mice are shown in Figure 2.

In agreement with previous studies (Kopp et al., 2004; Alexandre et al., 2008), we found that the power of "NREM sleep" produced by zolpidem in *C57BL/6J* mice was lower than that found in natural NREM sleep. Zolpidem reduced power during NREM sleep (Fig. 3*A*) over the frequency range between 5 and 16 Hz ($n = 5$, paired *t* test, $t_{(4)} = 4.5$, $p = 0.01$). In *γ2I77^{lox}* mice, zolpidem injection did not change the EEG power spectrum in either the waking (Fig. 3*B*) or NREM (Fig. 3*C*) states.

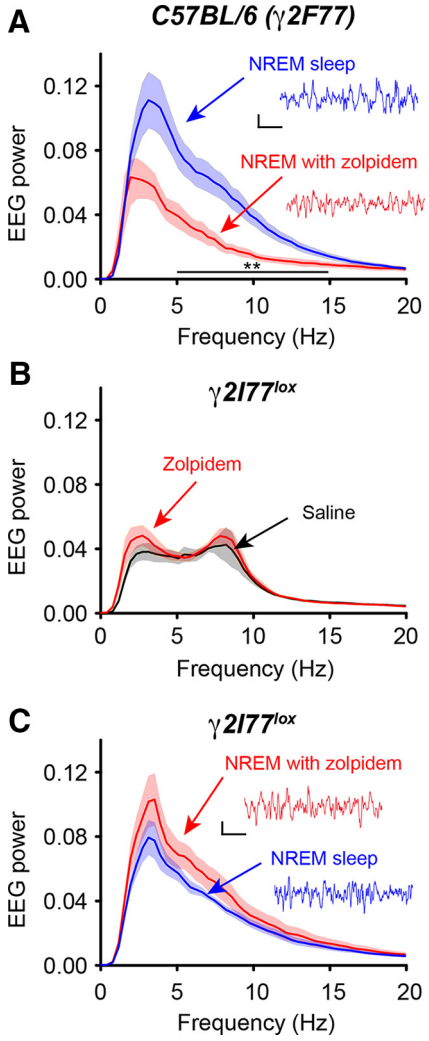


Figure 3. Zolpidem does not induce sleep in *γ2I77^{lox}* mice–EEG power analysis. ***A***, EEG power spectrum of natural NREM sleep (blue) or zolpidem-induced sleep (5 mg/kg, red) in *C57BL/6J(γ2F77)* mice. Zolpidem reduced power during NREM sleep (Fig. 3*A*) over the frequency range between 5 Hz and 16 Hz ($n = 5$, paired *t* test, $t_{(4)} = 4.5$, $p = 0.01$). ***B***, EEG power spectrum of *γ2I77^{lox}* mice ($n = 6$) in the waking state following saline intraperitoneal injection (black) or zolpidem (5 mg/kg i.p.; red). ***C***, In *γ2I77^{lox}* mice, zolpidem does not influence the power spectra during NREM sleep. Typical epochs of EEG trace are shown. Calibration: ***A***, ***C***, 200 μV, 500 ms.

Potentiation of GABA inputs onto histaminergic neurons by zolpidem induces and maintains NREM sleep

Having demonstrated that *γ2I77^{lox}* mice do not enter NREM sleep after systemic zolpidem administration, we next made several areas of the brains of *γ2I77^{lox}* mice zolpidem-sensitive using the γ2I77 to γ2F77 subunit switch. The first target was histamine neurons. Previously, we generated and studied *HDC-Δγ2I77* mice, which are mice with a deletion of the *γ2I77* subunit from histaminergic neurons in the TMN, obtained by crossing *HDC-Cre* and *γ2I77^{lox}* mice (Zecharia et al., 2012). The histaminergic neurons of these *HDC-Δγ2I77* mice lack IPSCs (Zecharia et al., 2012). Because the *HDC-Δγ2I77* mice still had Cre recombinase expressed in their histaminergic neurons, we could implement a "restorative genetics" strategy and put the γ2F77 subunit back into the neurons from which the γ2I77 version was deleted. We introduced the zolpidem-sensitive γ2F77 subunit into the histaminergic neurons of *HDC-Δγ2I77* mice using a Cre recombinase flex switch-dependent transgene (Atasoy et al., 2008), *flex-rev-*

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Figure 2. EEG spectra and sleep scoring for zolpidem-induced sleep in *C57BL/6 (γ2F77)* mice compared with γ2I77 ^{lox} mice. ***A***, EEG power spectra for *C57BL/6J* mice injected with saline or 5 mg/kg zolpidem. The spectra are aligned in register with the θ/δ power ratio, the root mean square electromyogram (RMS EMG), the primary EEG, and the sleep scoring assignments (Wake, NREM, REM). Arrow indicates the time of saline or zolpidem injection. ***B***, EEG power spectra for γ2I77 ^{lox} mice injected with saline or 5 mg/kg zolpidem. All alignments of traces are as above in ***A***.

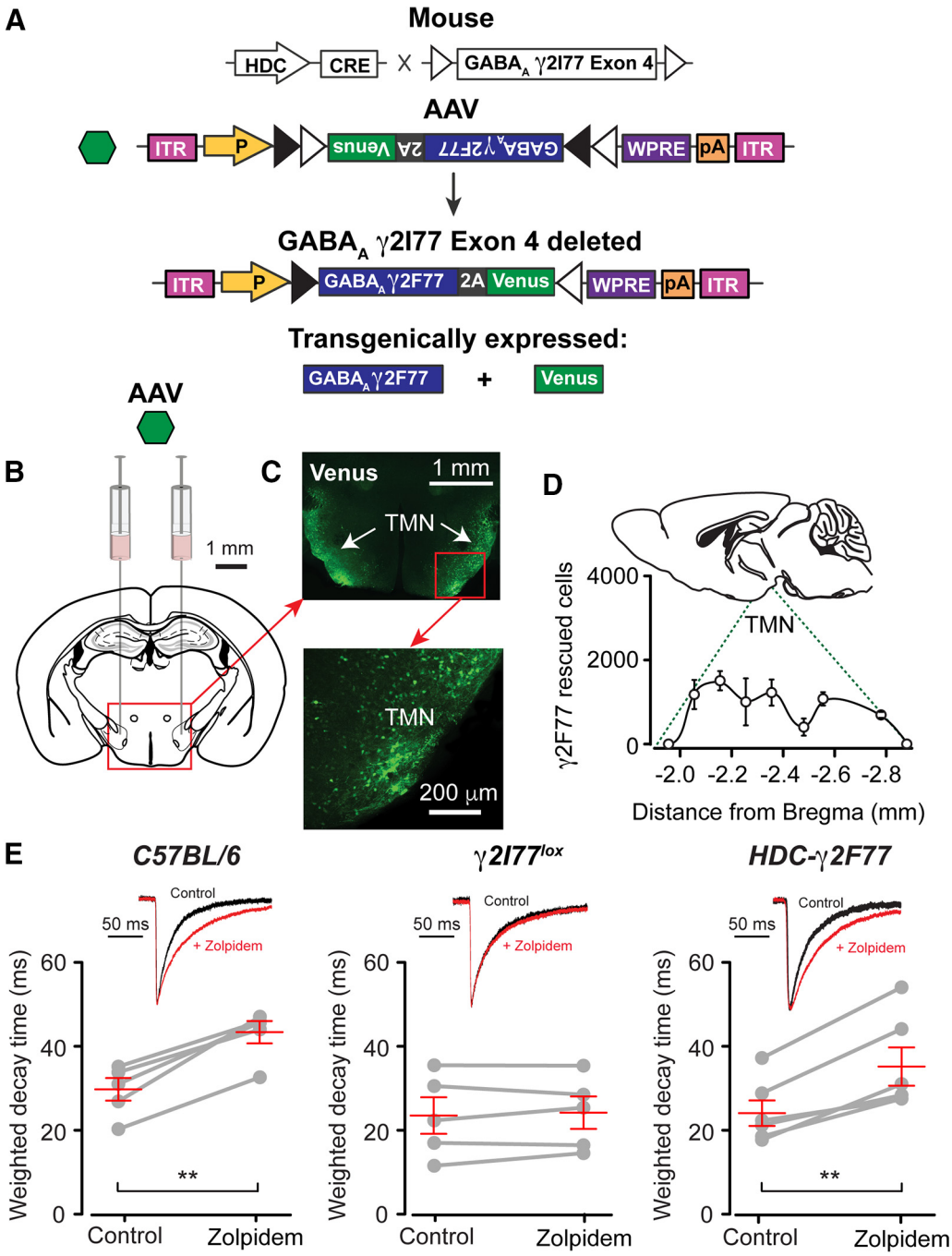
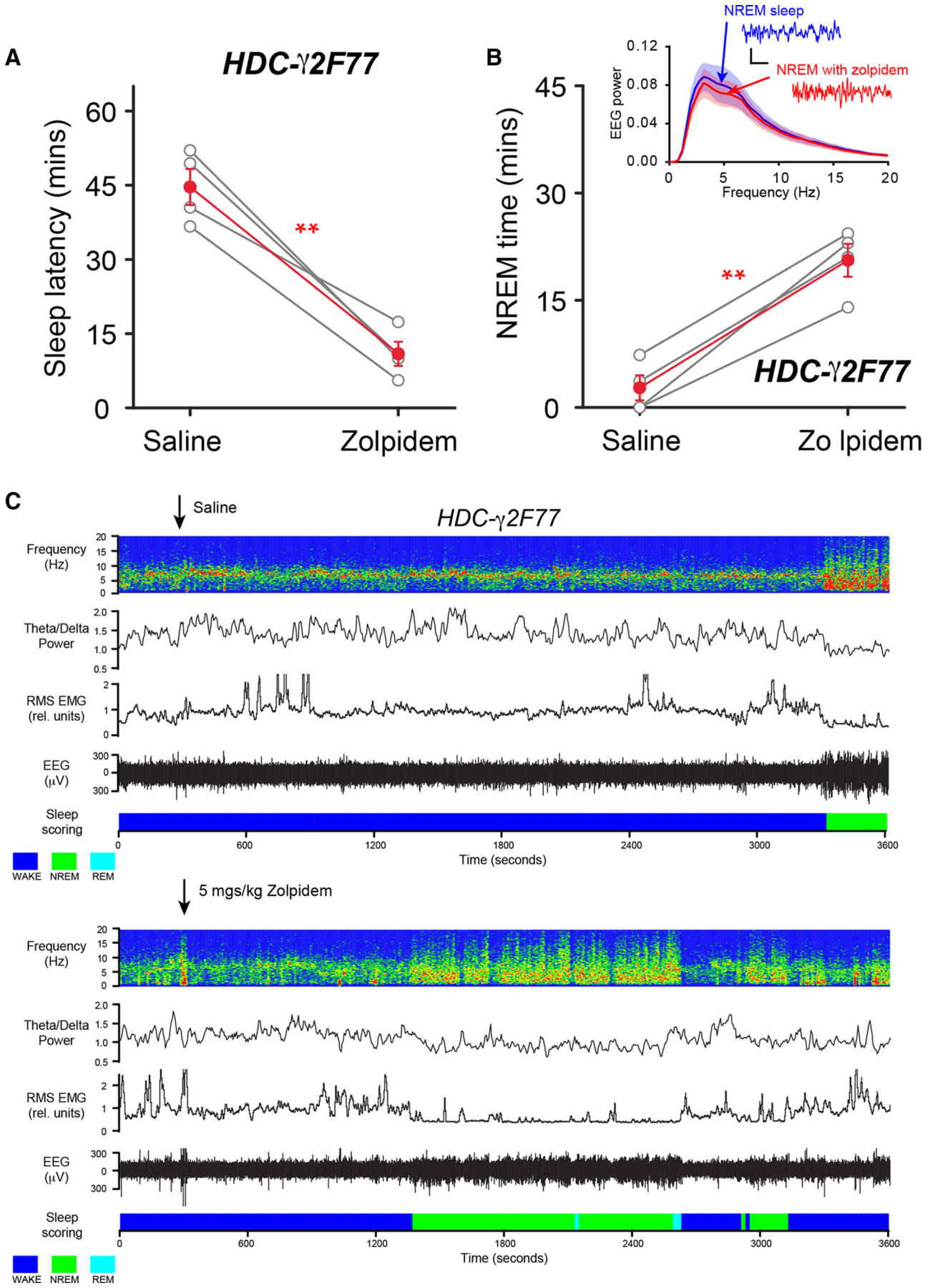


Figure 4. Making histamine neurons selectively sensitive to zolpidem. ***A***, Generating the *HDC-γ2F77* mice: the alleles *HDC-Cre* and *γ2I77^{lox}* in *HDC-Δγ2I77* mice, and the AAV flex-switch transgene construct *AAV-flex-rev-γ2F77-2A-Venus* used to selectively label (Venus) and introduce zolpidem sensitivity (γ2F77) in *HDC-Δγ2I77* neurons. Open triangles represent *loxP* sites. Filled triangles represent *lox2272* sites. ITR, Inverted terminal repeats; P, pan-promoter (*hdc* gene promoter fragment); WPRE, woodchuck post-transcriptional regulatory element. ***B***, Schematic of AAV microinjection into the TMN area of *HDC-Δγ2* mouse brain. ***C***, Histaminergic neurons labeled by Cre-dependent activation of the *AAV-flex-rev-γ2F77-2A-Venus* transgene, detected by immunocytochemistry with EGFP antisera. ***D***, Number of Venus-labeled neurons along the rostrocaudal axis of the posterior hypothalamus, detected by EGFP immunocytochemistry. ***E***, Spontaneous IPSCs recorded from histaminergic neurons in acute brain slices prepared from the posterior hypothalamus of *C57BL/6J* (*γ2F77*), *γ2I77^{lox}*, and *HDC-γ2F77* mice before (black) and after 10 μM zolpidem (red) application. Traces represent peak-normalized, superimposed average waveforms. For the *C57BL/6J* (*γ2F77*) data, 87 events were averaged before zolpidem and 61 events were averaged after zolpidem. For the γ2I77^{lox} data, 124 events were averaged before zolpidem and 167 events were averaged after zolpidem. For the *HDC-γ2F77* data, 59 events were averaged before zolpidem and 90 events were averaged after zolpidem. Graphs represent the mean weighted decay times before and after zolpidem application for the different groups of mice. $^{**}p = 0.002$ (paired *t* test).

γ2F77-2A-Venus, packaged into AAV capsids (Fig. 4*A*). This *AAV-flex-rev-γ2F77-2A-Venus* was bilaterally injected into the ventral TMN area of adult *HDC-Δγ2I77* mice to generate *HDC-γ2F77* mice (Fig. 4*B*). The flex switch in the AAV transgene ensured that γ2F77 expression was restricted to Cre-positive neurons (Fig. 4*A*). These mice had bilateral expression of the γ2F77-2A-Venus transgene in their TMN area, confined to HDC neurons in the ventral parts of the TMN (Fig. 4*B*,*C*), in an ~700 μm anterior-to-posterior segment (Fig. 4*D*). The mean number of AAV-transduced neurons in *HDC-γ2F77* brains, as assessed by Venus expression, was compared with a count performed on a brain from an *HDC-Cre x Rosa-YFP* reporter mouse cross. We


A
HDC-γ2F77
Sleep latency (mins)
60
45
30
15
0
**
Saline
Zolpidem
B
NREM sleep
NREM with zolpidem
EEG power
0.12
0.08
0.04
0.00
0
5
10
15
20
Frequency (Hz)
NREM time (mins)
45
30
15
0
**
HDC-γ2F77
Saline
Zo lpidem
C
Saline
HDC-γ2F77
Frequency (Hz)
Theta/Delta Power
RMS EMG (rel. units)
EEG (μV)
Sleep scoring
0
600
1200
1800
2400
3000
3600
Time (seconds)
WAKE
NREM
REM
5 mgs/kg Zolpidem
Frequency (Hz)
Theta/Delta Power
RMS EMG (rel. units)
EEG (μV)
Sleep scoring
Time (seconds)
WAKE
NREM
REM

found *HDC-Cre x Rosa-YFP* mice had ~8000 neurons in which the HDC promoter was active in the TMN area (data not shown). This could be an overestimate if some of the *Rosa-YFP* expression originates from the *HDC-Cre* gene turning on and off during development. In the adult *HDC-γ2F77* mice, where Venus expression can only be seen if the *HDC-Cre* gene is active in the adult, ~7000 Venus-positive cells could be detected. This number will be an underestimate because it cannot be expected that all histaminergic neurons in *HDC-Δγ2I77* mice would be transduced by the *AAV-flex-rev-γ2F77-2A-Venus* virus (see below). On the other hand, counts of cells immunoreactive for histamine estimated that there were 2500–3500 such neurons in the mouse hypothalamus (Parmentier et al., 2002), and 3800 in the rat determined by staining with histidine decarboxylase antibodies (Ericson et al., 1987). Given the difference in sensitivity between the genetic and primary immunoreactive detection methods, our estimate of histaminergic neuronal number is approximately in the same range. The extent of AAV transduction in *HDC-γ2F77* mice was also ascertained by whole-cell voltage-clamp recordings in acute slices made from the posterior hypothalamus. In the ventral TMN region, we found that 10 of 16 neurons (62.5%) had restored IPSCs that resembled IPSCs recorded from *C57BL/6J* neurons (e.g., Fig. 4*E*). Of these 16 cells, the remaining 6 still had no IPSCs (data not shown). Presumably, these six neurons had not been transduced by *AAV-flex-rev-γ2F77-2A-Venus* virus and were still *HDC-Δγ2I77* knock-out cells (Zecharia et al., 2012). We confirmed that the *HDC-γ2F77* histaminergic neurons with rescued IPSCs also had restored zolpidem sensitivity. Zolpidem (10 μM) applied to *C57BL/6 (γ2F77)* histaminergic cells slowed the IPSC decay by 48 ± 8%, from 30 ± 3 ms (control) to 44 ± 3 ms (zolpidem) ($n = 5$ cells, paired t test, $t_{(4)} = 7.5$, $p = 0.002$; Fig. 4*E*, left); by contrast, 10 μM zolpidem applied to $\gamma 2I77^{lox}$ histaminergic neurons had no effect on the IPSC decay, being ~23 ± 4 ms (control) and 24 ± 4 ms (zolpidem) ($n = 5$ cells, paired t test, $t_{(4)} = 0.7$, $p = 0.27$; Fig. 4*E*, middle). In contrast, 10 μM zolpidem applied to *HDC-γ2F77* TMN neurons slowed the IPSC decay by ~46 ± 7% from 24 ± 3 ms (control) to ~35 ± 5 ms (zolpidem) ($n = 6$ cells, paired t test, $t_{(5)} = 5.7$, $p = 0.002$; Fig. 4*E*, right), the same magnitude of response obtained by applying zolpidem to *C57BL/6J* histaminergic neurons (Fig. 4*E*, left).

The baseline sleep–wake parameters of *HDC-γ2F77* mice did not differ from $\gamma 2I77^{lox}$ or *C57BL/6J* mice (Table 1). We next examined whether administering zolpidem systemically to *HDC-γ2F77* mice induced sleep (Fig. 5). Following a zolpidem (5 mg/kg, i.p.) injection, the sleep latency of *HDC-γ2F77* mice was significantly decreased by ~75 ± 6%, from 45 ± 4 min (saline) to 11 ± 2 min (zolpidem) ($n = 4$; paired t test, $t_{(3)} = 8$, $p = 0.004$] (Fig. 5*A*). *HDC-γ2F77* mice spent significantly more time in NREM sleep (21 ± 2 min) compared with mice injected with saline (2.8 ± 1.8 min) ($n = 4$; paired t test, $t_{(3)} = 9.5$, $p = 0.002$; Fig. 5*B*). However, in the first 45 min after drug injection, zolpidem-injected *HDC-γ2F77* mice slept for approximately half as much time as zolpidem-injected *C57BL/6J* mice. Furthermore, in contrast to zolpidem's effects in *C57BL/6J* mice, zolpidem's ability to prolong sleep time in *HDC-γ2F77* did not persist beyond the first 45 min after injection. So we did not create the full effect of zolpidem (5 mg/kg) in *HDC-γ2F77* mice. In the sleep states induced by zolpidem in the *HDC-γ2F77* mice, EEG power in the zolpidem-induced sleep was not significantly reduced compared with that occurring in natural NREM sleep ($n = 4$; paired t test, 2–16 Hz inclusive, $t_{(3)} = 1.84$, $p = 0.16$; Fig. 5*B*, inset), which contrasts with the effect of zolpidem on EEG power in *C57BL/6J* mice (Fig. 3*A*). Examples of the primary EEG recordings and sleep-scoring for saline- and zolpidem-injected *HDC-γ2F77* mice are shown in Figure 5*C*.

The FC also can contribute to zolpidem-induced sleep induction but not maintenance

In some circumstances, the FC could help initiate sleep (see Discussion). To test whether zolpidem might work partly through this route to induce sedation, we made the FC of $\gamma 2I77^{lox}$ mice selectively zolpidem-sensitive by genetically swapping zolpidem-sensitive γ2F77 subunits into $\gamma 2I77^{lox}$ frontal cortical neurons, so generating *FC-γ2F77* mice (Fig. 6*A*,*B*). For this, we coinjected bilaterally a mixture of two AAVs into the FC: *AAV-Cre-2A-γ2F77* and *AAV-flex-rev-EGFP*. This produced cotransduced neurons. The swap works as follows. From the *AAV-Cre-2A-γ2F77* transgene, the Cre recombinase destroys production of functional γ2I77, and the zolpidem-sensitive γ2F77 subunit replaces it; the second AAV expresses EGFP only if Cre recombinase is present, and thus marks neurons that have been transduced with *AAV-Cre-2A-γ2F77* (Fig. 6*A*). We visualized the transduced area by serial sectioning and then immunocytochemistry with GFP antisera (Fig. 6*C*,*D*). In all injections, the spread of transduced cells reached rostral almost to the olfactory bulb and caudal as far as the primary motor cortex (Fig. 6*D*). *AAV-Cre-2A-γ2F77* transgene expression was found in both the frontal motor cortex and the prefrontal cortical areas (including prelimbic cortex, orbital areas, primary and secondary motor cortices). Maximally 20% of any given cortical area was transduced, mostly restricted to the deep layers of cortex, V and VI (Fig. 6*C*,*D*). To confirm that the γ2I77 to γ2F77 swap had produced zolpidem sensitivity in pyramidal neurons of *FC-γ2F77* mice, we made acute slices of M1 and M2 frontal neocortex and performed whole-cell voltage-clamp recordings on pyramidal neurons from layers 5 and 6. The neurons expressing the *AAV-Cre-2A-γ2F77* transgene were identified by their primary EGFP signal. Zolpidem (1 μM) slowed the decay of IPSCs by 63.5 ± 14% in EGFP-expressing neurons, from 15.4 ± 1.8 ms (control) to 24.5 ± 2.2 ms (zolpidem) ($n = 6$, paired t test, $t_{(5)} = 6.4$, $p = 0.001$) (Fig. 6*E*, right), which was similar to its effect on the IPSCs of *C57BL/6* pyramidal neurons ($n = 6$, paired t test, $t_{(5)} = 8.8$, $p = 0.0003$; Fig. 6*E*, left); by contrast, the decay of IPSCs was unchanged by zolpidem (13.2 ± 2.2 ms) in $\gamma 2I77^{lox}$ neocortical pyramidal neurons compared with control solution (12.8 ± 2.2 ms) ($n = 5$, paired t test, $t_{(4)} = 1$, $p = 0.4$; Fig. 6*E*, middle).

The baseline sleep–wake parameters of *FC-γ2F77* mice did not differ from $\gamma 2I77^{lox}$ or *C57BL/6J* mice (Table 1). We tested whether making FC neurons selectively zolpidem-sensitive was sufficient to produce a behavioral response in *FC-γ2F77* mice (Fig. 7). We administered zolpidem (5 mg/kg) to *FC-γ2F77* mice and found that this reduced latency to NREM sleep (Fig. 7*A*). *FC-γ2F77* mice had a 50 ± 14% shorter latency to NREM sleep (zolpidem: 13 ± 4 min) compared with those injected with saline

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Figure 5. Selective potentiation of GABA inputs onto histaminergic neurons allows zolpidem to induce and maintain sleep. ***A***, Sleep latencies after saline and zolpidem (5 mg/kg) injections in *HDC-γ2F77* mice. $^{**}p = 0.004$ (paired t test). ***B***, Time spent in NREM sleep in the first 45 min after saline and zolpidem in *HDC-γ2F77* mice. $^{**}p = 0.002$. ***B***, Inset, Normalized EEG power spectra from *HDC-γ2F77* mice following zolpidem (5 mg/kg i.p.; red) compared with their natural NREM sleep spectra (blue). Typical epochs of EEG trace are shown. ***C***, EEG power spectra for *HDC-γ2F77* mice injected with saline or 5 mg/kg zolpidem. The spectra are aligned in register with the θ/δ power ratio, the root mean square electromyogram (RMS EMG), the primary EEG, and the sleep scoring assignments (Wake, NREM, REM). Arrow indicates the time of saline or zolpidem injection.

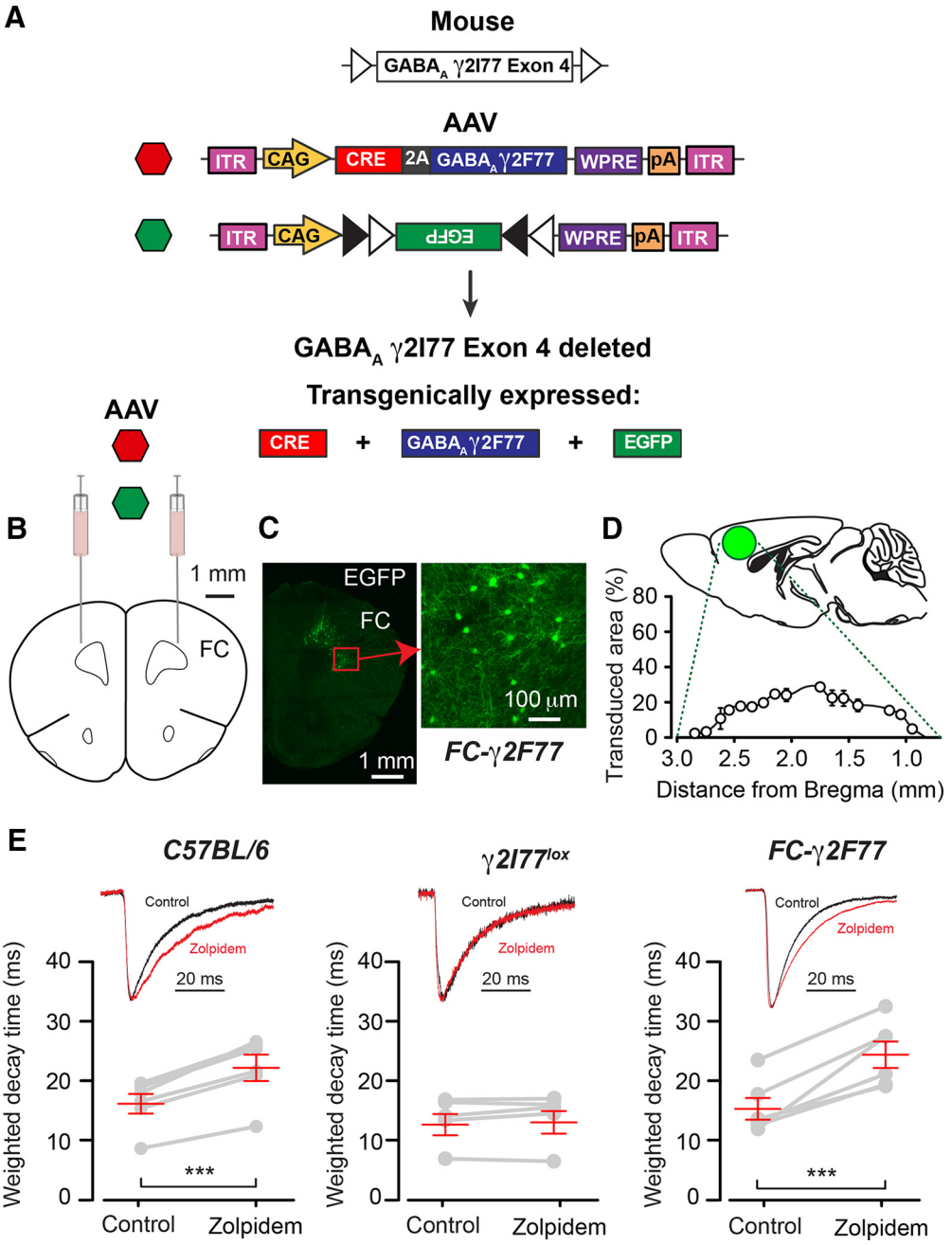

Figure 6. Making frontal cortical neurons selectively sensitive to zolpidem. ***A***, Generating the *FC-γ2F77* mice: the *γ2I77^{lox}* allele and the two AAVs, *AAV-Cre-2A-γ2F77* and *AAV-flex-rev-EGFP*, used to make the zolpidem-insensitive γ2I77 to zolpidem-sensitive γ2F77 subunit swap in γ2I77^{lox} mice in the FC. CMV, Cytomegalovirus enhancer/promoter; ITR, inverted terminal repeats; pA, polyadenylation signal; WPRE, woodchuck post-transcriptional regulatory element. ***B***, The two AAVs were mixed and coinjected into the FC of *γ2I77^{lox}* mice. ***C***, Overall expression of the *AAV-Cre-2A-γ2F77* transgene in pyramidal neurons (e.g., in layer V shown here) was detected by EGFP immunocytochemistry. ***D***, Percentage area of transduced frontal neocortical sites along the rostrocaudal axis. ***E***, Peak normalized spontaneous IPSCs recorded from cortical pyramidal neurons before (black) and after 1 μM zolpidem (red) from *C57BL/6J (γ2F77)*, *γ2I77^{lox}*, and *FC-γ2F77* neurons. Traces are superimposed averages. To construct these, 251 events (before zolpidem) and 175 events (after zolpidem) were averaged for *C57BL/6J* neurons; 74 events (before zolpidem) and 71 events (after zolpidem) were averaged for γ2I77^{lox} neurons; 247 events (before zolpidem) and 155 events (after zolpidem) were averaged for *HDC-γ2F77* neurons. Graphs represent mean weighted decay times of IPSCs before and after 1 μM zolpidem in *C57BL/6J* (γ2F77; ***$p = 0.0003$, paired *t* test), γ2I77^{lox} and *FC-γ2F77* (layer 5/6) pyramidal neurons (***$p = 0.001$, paired *t* test).

(30 ± 6 min) (Fig. 7*A*) ($n = 6$; paired *t* test, $t_{(5)} = 2.6$, $p = 0.045$). However, zolpidem did not significantly increase NREM time in *FC-γ2F77* mice compared with those injected with saline ($n = 6$; paired *t* test, $t_{(5)} = 1.5$, $p = 0.2$; Fig. 7*B*). The EEG power of this sleep following zolpidem administration in *FC-γ2F77* mice did not differ from natural NREM sleep ($n = 6$, paired *t* test, 2–16 Hz inclusive, $t_{(5)} = 0.95$, $p = 0.39$; Fig. 7*C*). Examples of the primary EEG recordings and sleep-scoring for saline- and zolpidem-injected *FC-γ2F77* mice are shown in Figure 8.

The SC do not contribute to zolpidem's sleep-inducing actions

Can zolpidem work in any brain area to induce NREM sleep? To test this, we looked at the SC (Fig. 9). The SCs are not associated with induction or maintenance of NREM sleep but do regulate eye movements and visual attention and also communicate with REM sleep-promoting areas. We made the SC of *γ2I77^{lox}* mice selectively zolpidem-sensitive. *SC-γ2F77* mice were generated by bilaterally injecting *AAV-Cre-2A-γ2F77* into the *SC* of *γ2I77^{lox}*

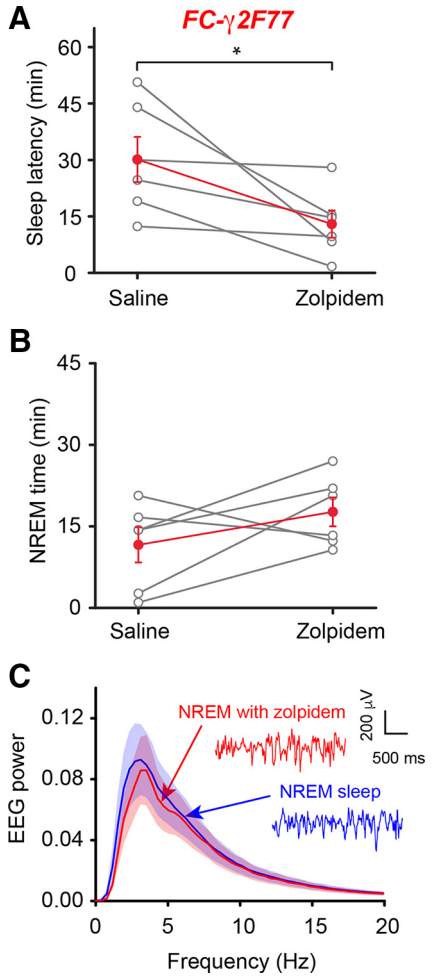

Figure 7. The FC contributes to zolpidem's ability to reduce sleep latency. ***A***, Sleep latencies. ***B***, Sleep times in the first 45 min after either saline or zolpidem (5 mg/kg) for *FC-γ2F77* mice. *$p = 0.045$ (paired *t* test). ***C***, Normalized EEG power spectrum from *FC-γ2F77* mice following zolpidem (5 mg/kg i.p.; red) compared with their natural NREM sleep spectrum (blue).

mice (Fig. 9*A*,*B*). *AAV-Cre-2A-γ2F77* transgene expression, detected by staining with Cre recombinase antisera (Fig. 9*C*), was found throughout the layers of the SC. The baseline sleep–wake parameters of *SC-γ2F77* mice did not differ from *γ2I77^{lox}* or *C57BL/6J* mice (Table 1). After zolpidem (5 mg/kg, i.p.) injection, the latency to NREM sleep (44 ± 10 min) of *SC-γ2F77* mice did not differ from that following a saline injection (37 ± 2 min) (Fig. 9*E*; $n = 4$; paired *t* test, $t_{(3)} = 0.8$, $p = 0.48$). The NREM sleep time of *SC-γ2F77* mice was also not increased during the first 45 min after zolpidem administration compared with saline ($n = 4$; paired *t* test, $t_{(3)} = 0.07$, $p = 0.95$; Fig. 9*F*). Zolpidem did not change the proportion of REM sleep in *SC-γ2F77* mice in the first 45 min after injection.

Discussion

We have previously suggested that sedatives produce sleep by interacting with the NREM sleep-inducing circuitry, changing activity in the hypothalamic and brainstem circuits that globally govern arousal (Nelson et al., 2002; Franks, 2008; Lu et al., 2008; Zhang et al., 2015). We show here that this seems to be the case for zolpidem, too. By using a pharmacogenetic method that probes endogenous GABA tone, we found that selectively augmenting the active GABA input onto hypothalamic histamine neurons by systemic zolpidem administration decreased NREM sleep latency and enhanced sleep time but without reducing power in the EEG. As well as revealing a potential site for zolpidem's sleep-promoting actions *in vivo*, our pharmacogenetic findings support the hypothesis that the initiation of natural NREM sleep could arise by increased and sustained inhibition onto histaminergic neurons (Nitz and Siegel, 1996; Sherin et al., 1996, 1998).

Clinical features of zolpidem mimicked in mice with brain regions selectively zolpidem-sensitive

Positive $GABA_A$ receptor modulators are often good at inducing sleep (Lancel and Steiger, 1999; Winsky-Sommerer, 2009; Nutt and Stahl, 2010; Rye et al., 2012). Zolpidem's pharmacokinetics make it effective for treating insomnia: it maximally occupies its receptor sites minutes after entering the blood, causing sleep quickly, but its short plasma half-life limits "hangovers" (Benavides et al., 1988). In controlled clinical settings, zolpidem's main effect on people is to reduce sleep latency; but overall zolpidem performs no better than placebo in sleep maintenance, wake time after sleep onset, or number of awakenings (Greenblatt and Roth, 2012). By these measures, zolpidem's key clinical action, reduction of sleep latency, is mimicked by increasing inhibition onto histaminergic neurons. However, the NREM sleep induced by zolpidem in humans and wild-type rodents does not entirely resemble natural sleep because "zolpidem sleep" has diminished power in the EEG compared with natural NREM sleep for frequencies >5 Hz in rodents (Kopp et al., 2004; Alexandre et al., 2008), and most frequencies in humans (Landolt et al., 2000). It is not clear whether this diminished EEG power is a good or bad feature of zolpidem-induced sleep. But in the *HDC-γ2F77* and *FC-γ2F77* mice, the power of zolpidem-evoked NREM sleep was the same as natural NREM sleep, so these "power-decreasing" effects of zolpidem must originate in other brain areas. This knowledge may be useful for designing sedatives that produce a more natural sleep.

Zolpidem can induce NREM sleep by selectively inhibiting histaminergic neurons

Despite having a 20-fold higher affinity at $\alpha1\beta\gamma2$-containing $GABA_A$ receptors (Pritchett and Seeburg, 1990), which are the most widely expressed and abundant type of $GABA_A$ receptors in the brain (Pritchett et al., 1989; Wisden et al., 1992; McKernan and Whiting, 1996), zolpidem (5 mg/kg) induces sleep through the $\alpha2\beta\gamma2$ and/or $\alpha3\beta\gamma2$ $GABA_A$ receptors (Kopp et al., 2004). The $\alpha1$-containing receptors are, instead, responsible for the decrease in EEG power across most frequencies >5 Hz in zolpidem-evoked sleep (Kopp et al., 2004). We might also expect that zolpidem's effects, such as sleep, result from additive slowing of IPSCs on cell types with $\alpha2$ and/or $\alpha3$ subunits throughout the brain. But this is not the case. Prolonging IPSCs on just histaminergic neurons is enough to induce and maintain sleep, although not to the full extent generated by zolpidem in wild-type C57BL/6J mice. Zolpidem is probably effective at histamine neurons because of their hub-like nature and their ability to promote arousal and wakefulness (Haas and Panula, 2003). Although there are relatively few histamine neurons, between 3000 and 7000 in the mouse, their axons ascend and descend from the TMN, coursing throughout the brain, coreleasing histamine and GABA to give balanced arousal (Wada et al., 1991; Haas and Panula, 2003; Yu et al., 2015). Thus, acutely inhibiting the "histamine hub" by zolpidem will cause histamine levels to fall throughout the brain and sleep to ensue. This fits with previous pharmacological data that infusing GABA agonists into the TMN area induces sleep (Lin et al., 1989; Nitz and Siegel, 1996), and

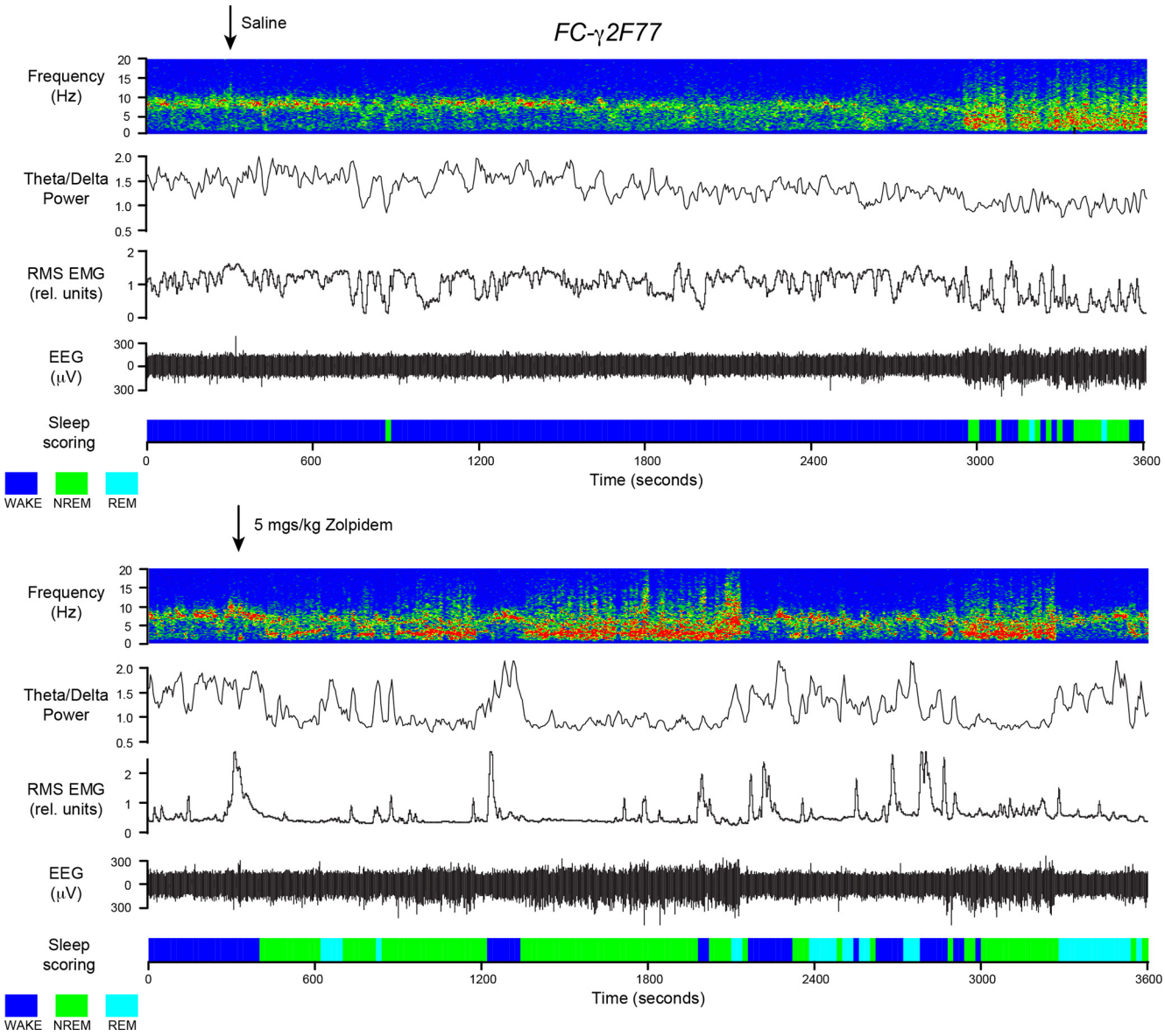

Figure 8. EEG power spectra and sleep scoring for zolpidem-induced sleep in *FC-γ2F77* mice. EEG power spectra for *FC-γ2F77* mice injected with saline or 5 mg/kg zolpidem. The spectra are aligned in register with the θ/δ power ratio, the root mean square electromyogram (RMS EMG), the primary EEG, and the sleep scoring assignments (Wake, NREM, REM). Arrow indicates the time of saline or zolpidem injection.

that GABA/galanin neurons in the lateral preoptic neurons, which send axons to the TMN, increase their activity during sleep (Sherin et al., 1996, 1998). Histaminergic neurons principally express $\alpha1\beta3\gamma2$ and $\alpha2\beta3\gamma2$ $GABA_A$ receptors (Fritschy and Mohler, 1995; Sergeeva et al., 2002; Zecharia et al., 2009, 2012; May et al., 2013). Thus, these $\alpha2$-containing $GABA_A$ receptors on the histaminergic neurons are likely candidates for a part of zolpidem's sleep-inducing actions *in vivo*. The $\alpha1$-containing $GABA_A$ receptors that cause zolpidem to reduce EEG power must be on other types of neurons elsewhere.

Zolpidem can initiate sleep top-down from the FC

We found that zolpidem can act in the frontal neocortex to reduce sleep latency, although the effect was not as large as for the histaminergic neurons, and sleep time was also not increased. Other data also link the frontal and preFC and behavioral sleep: sleep can initiate top-down if the FC is stimulated at 4 Hz (Penaloza-Rojas et al., 1964; Lineberry and Siegel, 1971); slow waves initiate in frontal neocortex (Massimini et al., 2004; Vyazovskiy et al., 2009); and in human aging, atrophy of the medial prefrontal cortex correlates with disrupted NREM slow waves (Mander et al., 2013).

Pharmacogenetic manipulation of GABA inputs versus receptor knock-outs, acute versus chronic

There are several caveats to consider when interpreting our results. The first point is that acute inhibition of one area in the brain could affect circuit dynamics in other areas (Otchy et al., 2015). The brain's dense interconnectivity could cloud, rather than reveal, the function of the inhibited region and so "transient circuit manipulations may have their own interpretive difficulties" (Otchy et al., 2015). This could indicate that zolpidem does not normally induce sleep by enhancing inhibition on histamine neurons but only does so in this particular artificial situation whereby the histamine neurons are made uniquely sensitive to zolpidem in the *HDC-γ2F77* mice. The second point is that dif-

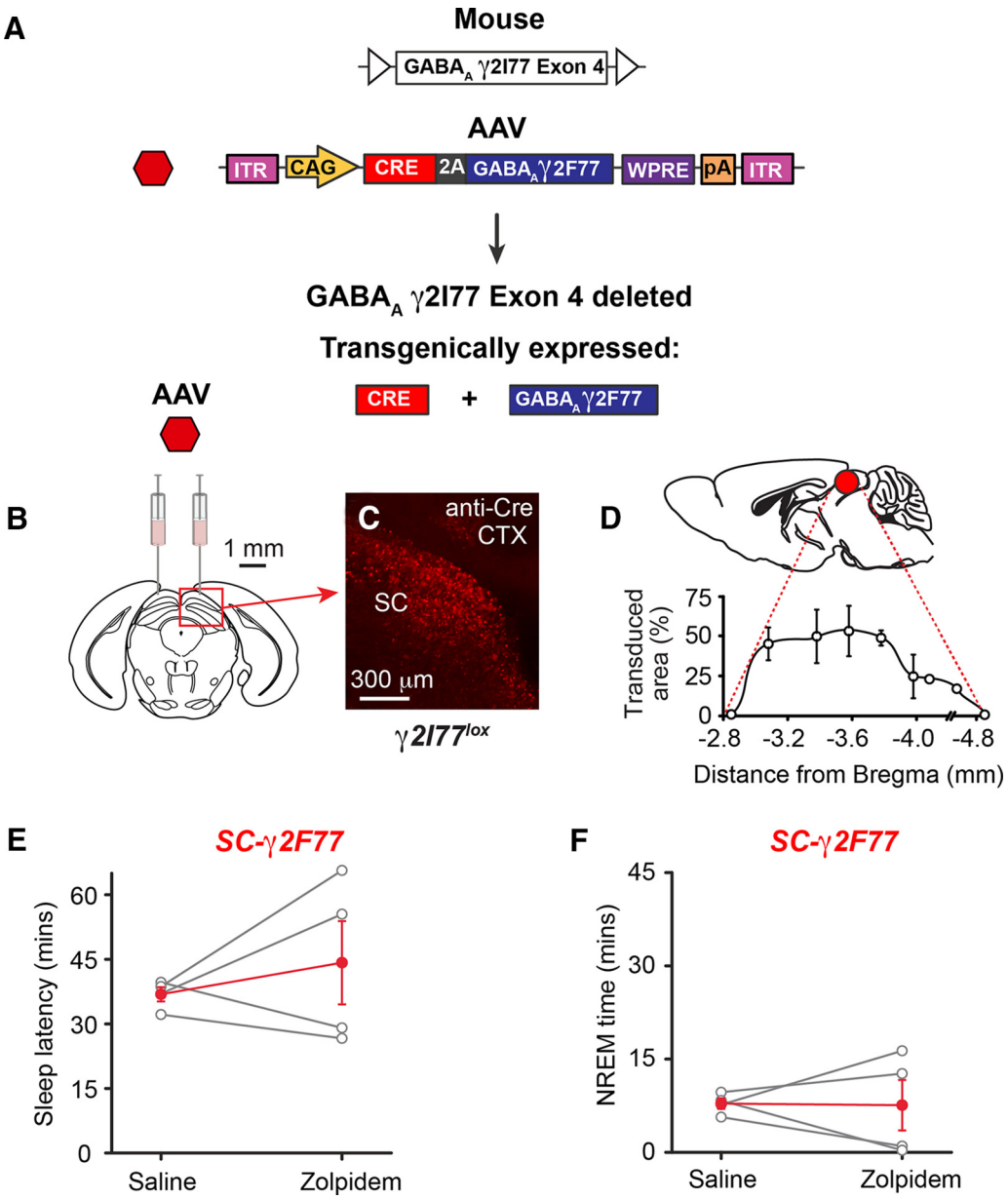

Figure 9. The SC do not contribute to zolpidem-induced sleep. ***A***, Generating the *SC-γ2F77* mice: the *AAV-Cre-2A-γ2F77* transgene used to make the zolpidem-insensitive *γ2I77* to zolpidem-sensitive *γ2F77* subunit swap in *γ2I77^{lox}* mice. CTX, Neocortex; CMV, cytomegalovirus enhancer/promoter; ITR, inverted terminal repeats; pA, polyadenylation signal; WPRE, woodchuck post-transcriptional regulatory element. ***B***, Bilateral *AAV-Cre-2A-γ2F77* microinjection into the SC of *γ2I77^{lox}* mouse brain to generate *SC-γ2F77* mice. ***C***, Detection of *AAV-Cre-2A-γ2F77* transgene expression in the SC by anti-Cre immunohistochemistry. CTX, Neocortex. ***D***, Percentage area of AAV-transduced SC along the rostrocaudal axis, deduced by counting Cre-positive cells. ***E***, Sleep latency. ***F***, Sleep time after saline and zolpidem (5 mg/kg) injections in *SC-γ2F77* mice.

ferent results are often produced by chronic or acute ablations (Wisden et al., 2009; Otchy et al., 2015). Genetic ablation of the GABA$_A$ receptor γ2 subunit from histaminergic neurons did not affect normal sleep over a 24 h period, although it did produce the more subtle effect of preventing the mice settling down and going to sleep in a new environment; in other words, removing synaptic GABA$_A$ receptors from histaminergic neurons lengthened the latency to NREM sleep (Zecharia et al., 2012), and this fits with our new data that, going in the opposite direction, enhancing IPSCs with zolpidem on these neurons shortens the latency to NREM sleep. Nevertheless, it remains remarkable that fast GABA input to the histamine neurons is dispensable for controlling the basic sleep–wake cycle. Similar to our results on histamine neurons, we found that chronic ablation versus acute pharmacogenetic modulation of GABA inputs on cerebellar Purkinje neurons also produced different results: mice with zolpidem-sensitive GABA$_A$ receptors selectively expressed in Purkinje neurons had acute ataxia after being given zolpidem, and so we concluded that ongoing GABA input onto Purkinje cells modulates motor control (Wulff et al., 2007; Wisden et al., 2009); by contrast, knocking out the γ2 subunit selectively and permanently from Purkinje cells, and the consequent removal of fast synaptic responses to GABA, did not produce overt ataxia, but only a subtle deficit in limb coordination (Vinueza Veloz et al., 2015). To explain the large difference in behavioral phenotype produced by acute zolpidem modulation of Purkinje cells and chronic ablation of fast inhibitory input, we hypothesized that the cerebellar circuitry with chronically removed synaptic GABA input on Purkinje cells

had undergone adaptation (Wulff et al., 2007; Wisden et al., 2009). We think the weak phenotypes produced by γ2 subunit ablation from Purkinje cells and histaminergic cells, and the contrasting strong phenotypes obtained by acute manipulation with zolpidem are analogous: the pharmacogenetic "zolpidem method" unmasks the acute role for GABA in modulating histaminergic neurons, whereas *HDC-Δγ2* mice have undergone compensatory changes. Acute zolpidem manipulation in *HDC-γ2F77* mice produces the "true" result.

In conclusion, zolpidem has rather subtle effects on synaptic IPSCs. Typically, it prolongs them by ~50%. We might have expected that zolpidem induces sleep by potentiating IPSCs everywhere in the brain; the net effect would be behavioral sleep. But instead we have shown that zolpidem can induce sleep by strengthening GABA signaling on just one cell type (histamine neurons). Normally, the NREM sleep induced by zolpidem does not resemble natural sleep; the drug produces a lower power in most frequencies of the EEG during NREM sleep. But via histamine neurons, zolpidem can induce sleep without reducing the EEG power of the sleep. This knowledge could help design drugs that induce a more natural sleep.

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